Blockade of VEGF accelerates proteinuria, via decrease in nephrin expression in rat crescentic glomerulonephritis

A Hara[1], T Wada[1], K Furuichi[1], N Sakai[1], H Kawachi[2], F Shimizu[2], M Shibuya[3], K Matsushima[4], H Yokoyama[5], K Egashira[6] and S Kaneko[1]

[1]*Department of Gastroenterology and Nephrology, Graduate School of Medical Science, Kanazawa University, Kanazawa, Japan;* [2]*Department of Cell Biology, Institute of Nephrology, Graduate School of Medical and Dental Sciences, Niigata University, Niigata, Japan;* [3]*Department of Genetics, Institute of Medical Science, Tokyo, Japan;* [4]*Department of Molecular Preventive Medicine, Graduate School of Medicine, The University of Tokyo, Tokyo, Japan;* [5]*Division of Blood Purification, Kanazawa University, Kanazawa, Japan and* [6]*The Department of Cardiovascular Medicine, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan*

Vascular endothelial growth factor (VEGF) is a potent angiogenic factor that maintains the glomerular and peritubular capillary (PTC) network in the kidney. The soluble form of the VEGF receptor-1 (soluble fms-like tyrosine kinase 1 (sFlt-1)) is known to regulate VEGF activity by binding VEGF in the circulation. We hypothesized that VEGF may be beneficial for maintaining glomerular filtration barrier and vascular network in rats with progressive glomerulonephritis (GN). For blockade of VEGF activity *in vivo*, rats were transfected twice with plasmid DNA encoding the murine sFlt-1 gene into femoral muscle 3 days before and 2 weeks after the induction of antiglomerular basement membrane antibody-induced GN. Inhibition of VEGF with sFlt-1 resulted in massive urinary protein excretion, concomitantly with downregulated expression of nephrin in nephritic rats. Further, blockade of VEGF induced mild proteinuria in normal rats. Administration of sFlt-1 affected neither the infiltration of macrophages nor crescentic formation. In contrast, treatment of sFlt-1 accelerated the progression of glomerulosclerosis and interstitial fibrosis accompanied with renal dysfunction and PTC loss at day 56. VEGF may play a role in maintaining the podocyte function as well as renal vasculature, thereby protecting glomeruli and interstitium from progressive renal insults.

Kidney International (2006) **69,** 1986–1995. doi:10.1038/sj.ki.5000439; published online 26 April 2006

KEYWORDS: VEGF; proteinuria; podocyte; nephrin; anti-GBM disease

Correspondence: *T Wada, Department of Gastroenterology and Nephrology and Division of Blood Purification, Graduate School of Medical Science, Kanazawa University, 13-1 Takara-machi, Kanazawa 920-8641, Japan. E-mail: twada@medf.m.kanazawa-u.ac.jp*

Received 16 June 2005; revised 26 January 2006; accepted 15 February 2006; published online 26 April 2006

Vascular endothelial growth factor (VEGF) is a potent angiogenic factor that induces endothelial cell migration, growth, differentiation, and regeneration through its receptors, vascular endothelial growth factor receptor (VEGFR)-1 (Flt-1) and VEGFR-2 (KDR/Flk-1).[1] In kidney, VEGF is abundantly expressed in glomerular epithelial cells (podocytes) and tubular epithelial cells, whereas the glomerular and peritubular capillary (PTC) endothelial cells express cognate VEGF receptors.[2–4] The importance of VEGF in kidney is evidenced by the fact that deficiency in VEGF selectively in the podocytes showed impaired glomerular capillary formation owing to a loss of endothelial cells in mice,[5] and antagonizing circulating VEGF caused glomerular endotheliosis in pregnant rats, which is also noted in human pre-eclampsia.[6]

In addition to the impacts of VEGF on endothelial cells, physiological levels of VEGF are pivotal for maintaining glomerular filtration barrier. In this regard, Sugimoto *et al.* reported that anti-VEGF antibodies and soluble VEGF receptor 1 (soluble fms-like tyrosine kinase 1 (sFlt-1)), which inhibit VEGF activity by directly sequestering VEGF and by functioning as a dominant-negative inhibitor against VEGF receptors,[7] respectively, induced proteinuria associated with podocyte dysfunction in normal kidneys.[8] Recent reports from human clinical cancer trials using anti-VEGF antibodies (Bevacizumab) suggest that proteinuria may be associated with treatment protocols.[9–11]

Therefore, we hypothesized in this study that VEGF may be responsible for maintaining the glomerular filtration barrier and vascular network in rats with progressive glomerulonephritis (GN). Molecular and pathological mechanisms involved in the increased levels of urinary protein, electron microscopic findings, and the expression of slit diaphragm-associated molecules were examined. Further, the effect of inhibition of VEGF on podocyte-associated molecules was examined. The present study shows that blockade of VEGF activity by transfection of the plasmid DNA encoding sFlt-1

gene in femoral muscles resulted in massive urinary protein excretion, concomitantly with downregulated expression of nephrin, which is one of the major glomerular slit diaphragm-associated molecules,[12] in diseased glomeruli. Treatment with sFlt-1 affected the progression of glomerulosclerosis and interstitial fibrosis, resulting in renal dysfunction. These results suggest that VEGF may play a role in maintaining the podocyte function as well as renal vasculature, thereby regulating urinary protein excretion and protecting glomeruli and interstitium from progressive insults.

RESULTS

Expression of sFlt-1

Muscle fibers positive for sFlt-1 were observed in femoral muscles from the sFlt-1 gene-transfected rats 6 days after disease induction (Figure 1a). In contrast, sFlt-1 protein was not detected in the muscles from the empty plasmid-treated rats (Figure 1b). Further, reverse transcription-polymerase chain reaction (RT-PCR) analysis of muscle tissue for sFlt-1 mRNA showed that sFlt-1 mRNA was expressed at the injected sites in sFlt-1-treated rats (Figure 1c). Finally, urinary levels of sFlt-1 increased at day 6 in rats treated with sFlt-1 compared with those from vehicle-treated rats (Figure 1d), and remained elevated for at least 14 days (data not shown).

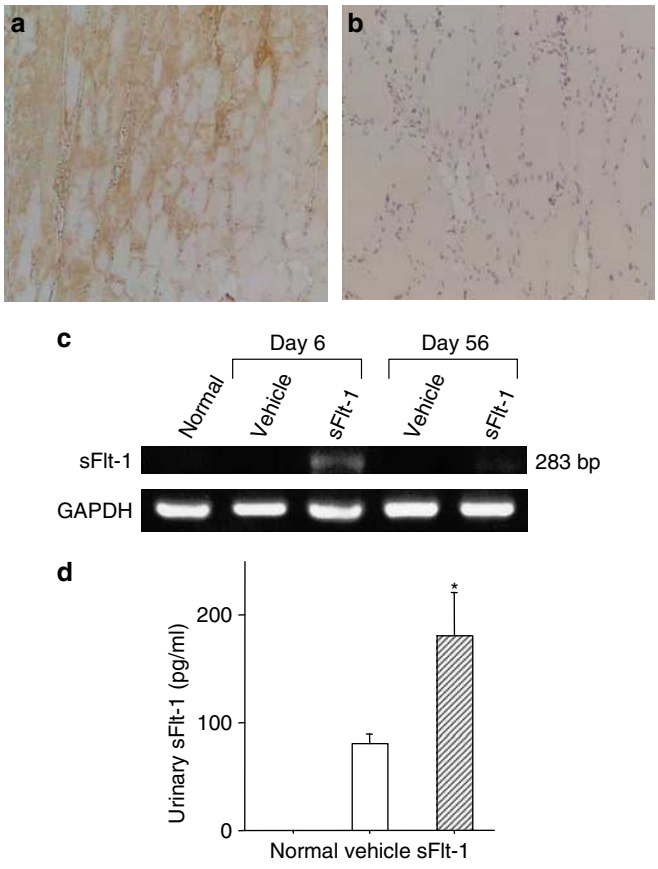

Figure 1 | sFlt-1 expression in femoral muscle. The expression of sFlt-1 in skeletal muscles, where gene transfer was performed with electroporation, was detected by an immunohistochemical method. (**a**) Fibers positive for sFlt-1 were observed in the femoral muscle from the plasmid DNA encoding sFlt-1-transfected rats 6 days after induction of GN. (**b**) There was no staining for sFlt-1 in the muscles from rats transfected with empty plasmid. sFlt-1 mRNA in muscles was determined by RT-PCR analysis. (**c**) sFlt-1 mRNA was detected in muscles 6 days after induction of GN, and barely detected 56 days after induction of GN. (**d**) Urinary levels of sFlt-1 increased 6 days after disease induction in sFlt-1-treated rats. Original magnification, × 100. *$P < 0.05$ compared with rats transfected with empty plasmid.

Histopathological studies

Semiquantitative evaluation of deposition showed no significant difference in the deposition of rabbit immunoglobulin (Ig)G, rat IgG, or rat C3 between glomeruli from rats administered sFlt-1 gene or empty plasmid only (data not shown). These results suggest that induction of GN was equivalent in the two groups.

Glomerular lesions showed endocapillary proliferation, severe necrotizing lesions, and crescentic formation at day 6. Numbers of ED-1-, proliferating cell nuclear antigen-, and CD8-positive cells did not significantly differ by the administration of sFlt-1 gene at day 6. Similarly, there was no significant difference between sFlt-1- and vehicle-treated rats in formation of crescentic lesions and the number of total glomerular cells at day 6 (Table 1).

Vehicle-treated nephritic animals showed a marked degree of glomerulosclerosis, as well as interstitial fibrosis at day 56 (Figure 2a–d). sFlt-1-treated rats showed significantly more glomerulosclerosis and interstitial fibrosis at day 56.

Effects of VEGF blockade on urinary protein excretion and renal function

Ten normal untreated rats excreted minute amounts of protein in the urine. Blockade of VEGF-induced mild proteinuria by day 6 in six normal rats without anti-GBM serum, which was maintained for 28 days, but returned to nearly normal levels at day 56 (Figure 3). In contrast, all nephritic rats injected with empty plasmid excreted markedly elevated amounts of protein in the urine at days 6, 14, and 56. The administration of sFlt-1 gene significantly increased proteinuria at days 14 and 56 compared with vehicle-treated rats (Figure 3). Nephritic rats with inhibition of VEGF showed hypoproteinemia, evidenced by reduced serum total protein levels and massive ascites at killing (Figure 4a). At day

Table 1 | Effects of inhibition of VEGF activity via sFlt-1 on histopathological changes on day 6[a]

	Normal	Vehicle	sFlt-1	*P*-value[b]
Crescentic formation (%)	0	37.3±2.7	36.4±5.4	0.88
Total cell number[c]	55.3±1.1	109.2±1.5	113.5±1.6	0.09
PCNA-positive cells[c]	1.8±0.5	7.0±0.2	7.1±0.4	0.88
ED-1-positive cells[c]	0.8±0.1	13.9±0.5	13.4±0.7	0.52
CD8-positive cells[c]	0.2±0.02	1.9±0.1	1.9±0.1	0.89

Abbreviations: PCNA, proliferating cell nuclear antigen; sFlt-1, soluble fms-like tyrosine kinase 1; VEGF, vascular endothelial growth factor.
[a]Values are given as mean±s.e.m. $n=4$ in normal group, $n=5$ in vehicle group, and $n=4$ in sFlt-1 group.
[b]Statistical analyses are based on unpaired Student's *t*-test and Kruskal–Wallis test.
[c]Number of cells/glomerular cross-section.

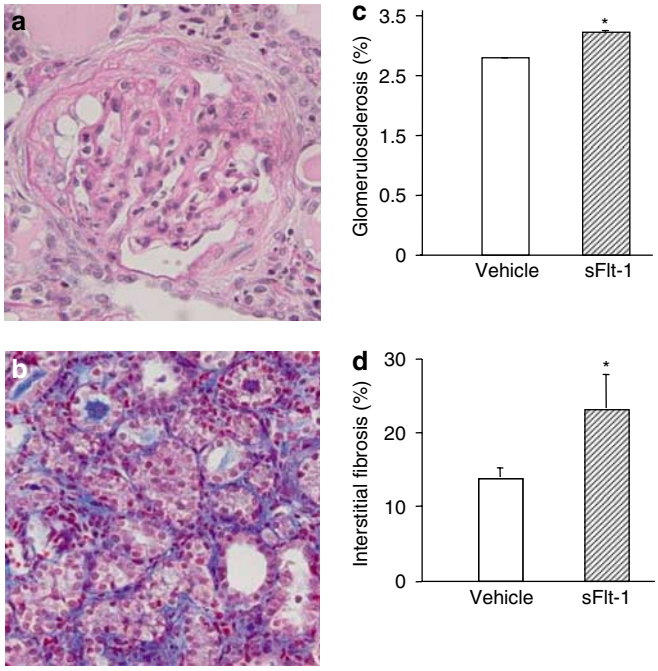

Figure 2 | VEGF inhibition accelerated glomerulosclerosis and interstitial fibrosis. Progressive renal lesions exhibited (**a**, **c**) glomerulosclerosis and (**c**, **d**) interstitial fibrosis in rats transfected with empty plasmid at day 56. In contrast, sFlt-1 treatment accelerated renal pathology. (**c**) Glomerulosclerosis and (**d**) interstitial fibrosis were more prominent. Original magnification, $\times 200$. $^{*}P<0.05$ compared with rats transfected with empty plasmid.

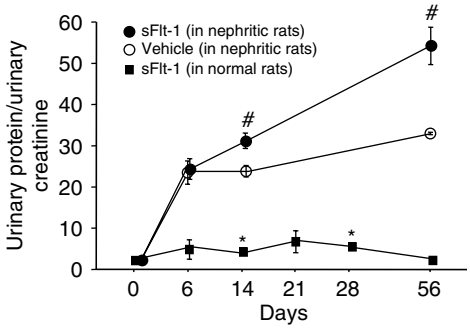

Figure 3 | Increased urinary protein excretion by inhibition of VEGF activity. Treatment of normal rats with sFlt-1 induced mild proteinuria, and its levels were maintained for 28 days and were decreased by (■) day 56. $^{*}P<0.01$ compared with a level of proteinuria at day 0. Results from rats administered with (○) empty plasmid and (●) sFlt-1. Administration of sFlt-1 markedly increased proteinuria. $^{\#}P<0.05$ compared with rats transfected with empty plasmid.

56, animals with empty plasmid developed renal dysfunction as evidenced by increased blood urea nitrogen levels. However, inhibition of VEGF by administration of sFlt-1 gene increased blood urea nitrogen levels (Figure 4b). Serum creatinine levels in sFlt-1-treated rats tended to increase compared with those in vehicle-treated rats at day 56, but the difference was not statistically significant.

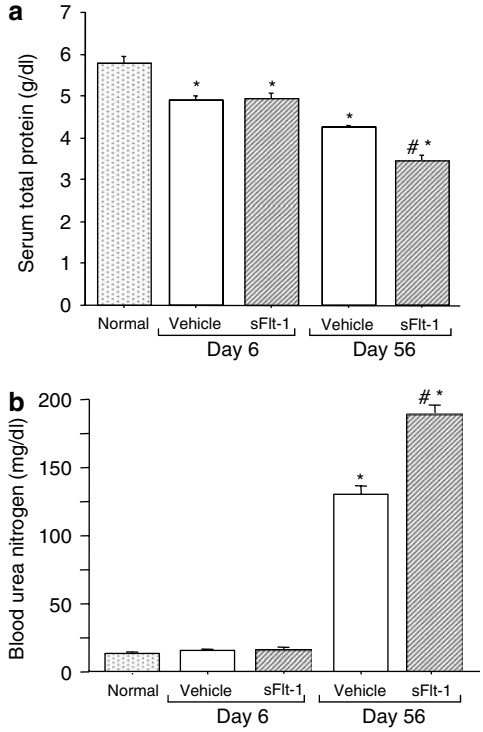

Figure 4 | Decreased serum total protein levels and deterioration of renal function by sFlt-1 treatment. (**a**) Serum total protein levels were decreased in sFlt-1-treated rats at day 56. $^{*}P<0.05$ compared with normal rats. $^{\#}P<0.05$ compared with vehicle-treated rats. (**b**) Rats treated with sFlt-1 developed renal dysfunction, evidenced by increased blood urea nitrogen levels at day 56. $^{*}P<0.001$ compared with normal rats. $^{\#}P<0.05$ compared with vehicle-treated rats.

Effects of VEGF blockade on glomerular epithelial cells and slit diaphragm-associated molecules

Epithelial foot processes tended to fuse in all tufts at day 6 in diseased glomeruli from rats treated with empty plasmid (Figure 5a; white arrow). In nephritic rats treated with sFlt-1, more severe fusion of epithelial foot processes occurred, and podocyte foot processes could be identified hardly (Figure 5b; black arrow). Podocytes in normal kidneys had faint desmin expression. In contrast, desmin was expressed by podocytes in nephritic rats and its expression was increased in sFlt-1-treated rats both at days 6 and 56 (Figure 5c and d).

mRNA expression of nephrin was specifically decreased by the inhibition of VEGF (Figure 5e). Nephrin protein, evaluated immunohistochemically in normal kidneys, was detected in a linear pattern along glomerular capillary walls (Figure 5f). Compared with rats treated with empty plasmid (Figure 5g), nephrin protein was reduced in sFlt-1-treated rats (Figure 5h). Concomitantly, Western blot analysis for nephrin protein revealed that nephrin was reduced in nephritic rats and that the reduction was more severe in rats treated with sFlt-1 (Figure 5i). In addition, nephrin was faintly expressed in both sFlt-1- and empty plasmid-treated rats at day 56.

In contrast to nephrin expression, expression of podocin, podoplanin, and podocalyxin did not change by the treat-

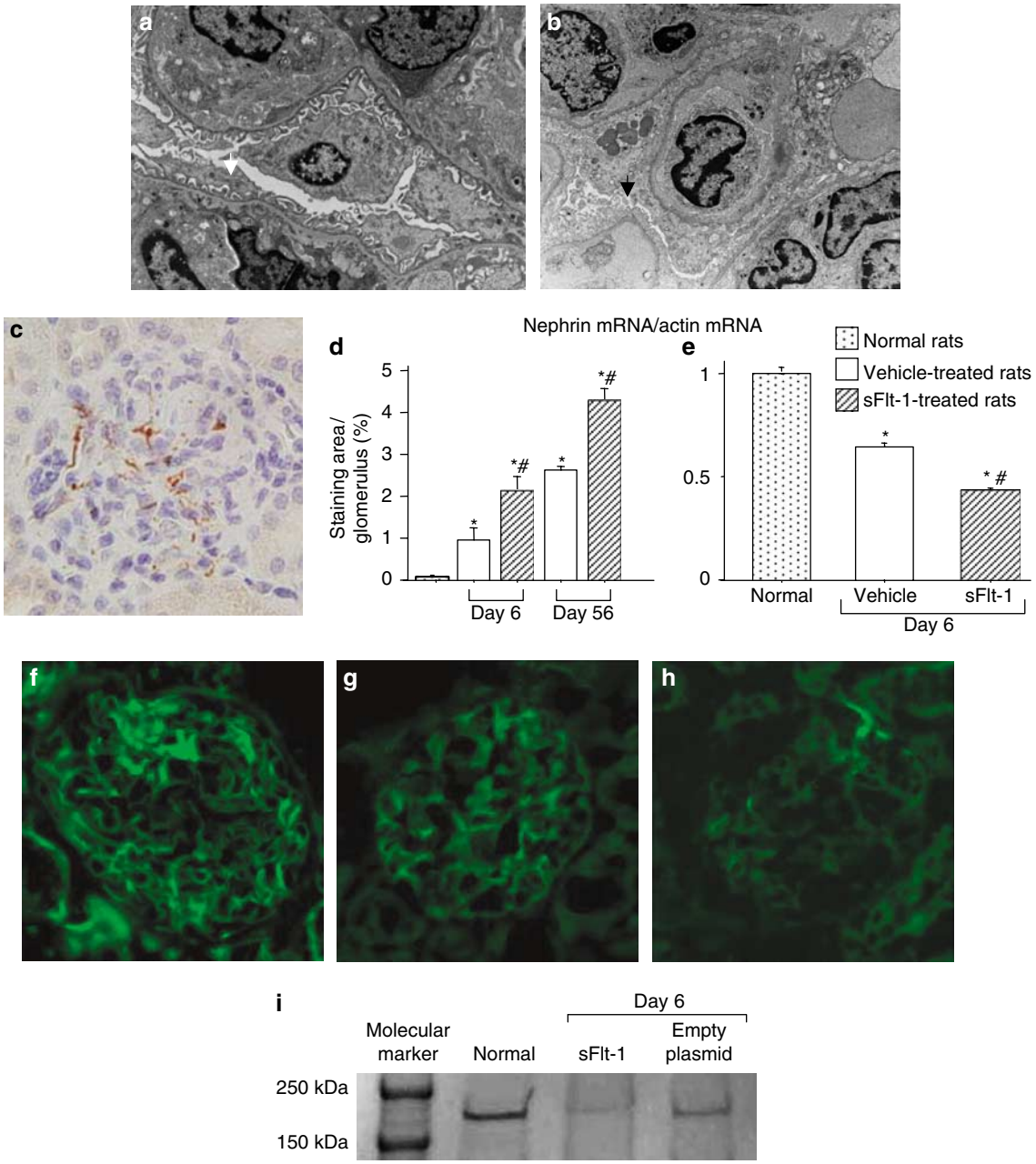

Figure 5 | Effects of anti-GBM GN and sFlt-1 treatment on podocytes and nephrin. (**a**) Podocyte foot processes fused in all tufts at day 6 in diseased glomeruli from rats transfected with empty plasmid. (**b**) sFlt-1 treatment accelerated fusion of podocyte foot processes. Original magnification, $\times$ 3000. (**c**) Expression of desmin at day 6 in diseased glomeruli from rats with empty plasmid. (**d**) Increased desmin expression in podocytes was observed in sFlt-1-treated rats at days 6 and 56. Original magnification, $\times$ 400. (**e**) mRNA expression of nephrin in normal and diseased kidneys. Expression of nephrin mRNA was decreased in anti-GBM GN and further downregulated by sFlt-1 treatment. $^{*}P<0.05$ compared with normal rats. $^{\#}P<0.05$ compared with vehicle-treated rats. Immunofluorescent microscopic findings of nephrin in (**f**) normal rats and (**g**) rats with empty plasmid. Nephrin expression was reduced at day 6. (**h**) Nephrin expression was further reduced in sFlt-1-treated rats compared with rats with empty plasmid at day 6. Original magnification, $\times$ 200. (**i**) sFlt-1 treatment markedly downregulated nephrin in diseased glomeruli as shown by Western blot analyses. Molecular markers are shown in the left lane. Data are representative of three experiments.

ment with sFlt-1 in either mRNA levels (Figure 6a–c) or protein levels (Figure 6d–g).

Microvascular changes associated with blockade of VEGF

In normal kidneys, thrombomodulin (TM)-positive glomerular and PTC endothelial cells were preserved (Figure 7a). Six days after disease induction, sFlt-1-treated rats (Figure 7c and d) had more severe TM-positive glomerular endothelial cell loss than that observed in rats injected with empty plasmid (Figure 7b and d). sFlt-1-treated rats tended to have decreased areas of TM-positive PTC endothelial cells compared with rats injected with empty plasmid at day 6 (Figure 7e).

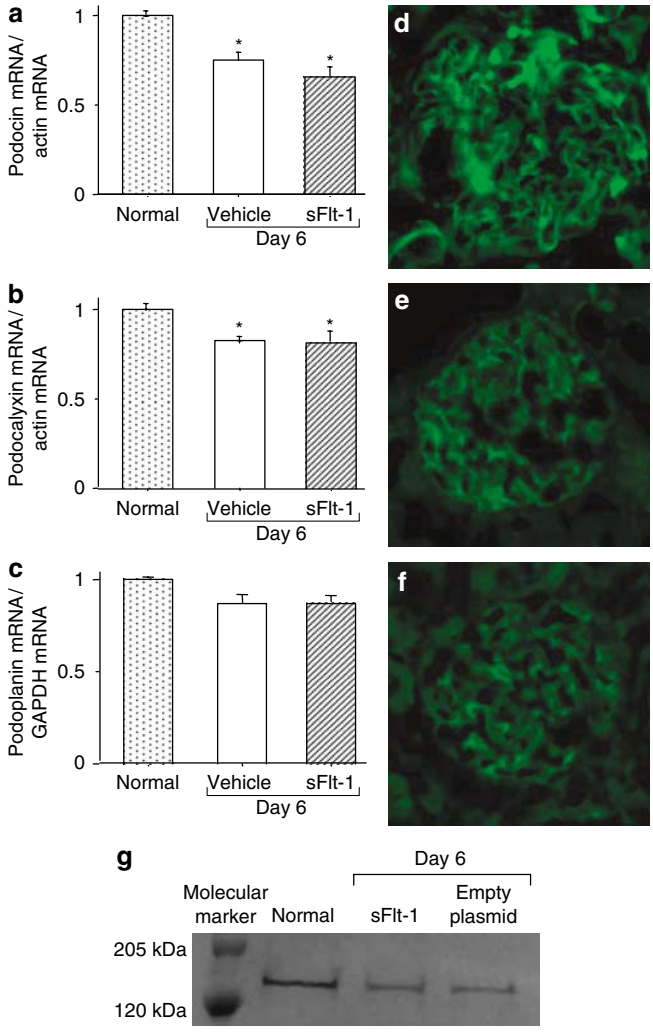

Figure 6 | Effects of anti-GBM GN and sFlt-1 treatment on podocyte-associated molecules. mRNA expression of (**a**) podocin, (**b**) podocalyxin, and (**c**) podoplaninwas decreased by anti-GBM GN and sFlt-1 treatment did not have impact on mRNA expression of these three podocyte-associated molecules. $^*P<0.05$ compared with normal rats. (**d**) Immunofluorescent microscopic findings of podocalyxin in normal rats. (**e**) Podocalyxin expression was reduced at day 6 in nephritic rats transfected with empty plasmid, which was not different from that in (**f**) sFlt-1-treated rats. Original magnification, $\times 200$. (**g**) sFlt-1 treatment did not alter the podocalyxin expression in diseased glomeruli revealed by Western blot analyses. Molecular markers are shown in the left lane. Data are representative of three experiments.

Expressions of VEGF and its receptors, Flk-1 and Flt-1, in normal and diseased kidneys

mRNA expression of three isoforms of VEGF in kidneys was reduced in crescentic GN, and was further reduced by the inhibition of VEGF (Figure 8a and b). In addition, these three isoforms of VEGF were only faintly expressed in both sFlt-1- and empty plasmid-treated rats at day 56 (Figure 8a and b). VEGF protein was detected on podocytes in normal kidneys by immunohistochemistry (Figure 8c). Compared with rats treated with empty plasmid (Figure 8d), sFlt-1-treated rats had reduced VEGF protein at day 6 (Figure 8e). VEGF receptor, Flk-1, was detected in all glomeruli and PTC endothelial cells in normal rat (Figure 8f). Six days after induction of nephritis, the area of glomerular and tubulointerstitial tissue expressions of Flk-1 was decreased compared with normal rats (Figure 8g, h, l and m). At day 56, Flk-1 expression in glomeruli and interstitium was reduced via VEGF blockade (Figure 8l and m). Similarly, the expression of Flt-1 in diseased kidneys was barely detectable, whereas it was readily detected in podocytes in normal kidneys (Figure 8i–k).

DISCUSSION

This study demonstrates that administration of sFlt-1, a selective and potent inhibitor of VEGF, accelerates proteinuria with massive ascites, glomerulosclerosis, and interstitial fibrosis in rat crescentic GN, and is associated with loss of a slit diaphragm-associated molecule, nephrin, and endothelium. In this study, sFlt-1 gene transfected in femoral muscles was expressed at injected sites at mRNA and protein levels and was released to circulation, from which it translocated to urinary space beyond the glomerular basement membrane (GBM) in diseased kidneys.[7,13] This study suggests that VEGF in rats with crescentic GN may be important for maintaining renal vasculature and preventing proteinuria via regulation of nephrin, VEGF appears to protect the kidney from renal insults.

Blockade of VEGF accelerated proteinuria and reduced specifically nephrin. Nephrin, a product of the NPHS1 gene whose mutations cause congenital nephrotic syndrome of the Finnish type, is exclusively expressed by glomerular podocytes within the kidney and is localized to the podocyte slit diaphragm.[12,14] Recently, it has been reported that nephrin not only is a key slit diaphragm component but also prevents podocyte apoptosis.[15] Mice injected with anti-VEGF antibody or sFlt-1 developed proteinuria accompanied by disruption/loss of slit diaphragm and specific nephrin downregulation.[8] Therefore, one explanation for decreased expression of podocyte-associated molecules is that as a result of anti-GBM antibody-induced GN, podocyte damage occurred and progressed, followed by decreased expression of nephrin. Moreover, treatment with VEGF results in decreased urinary protein excretion and renal dysfunction.[16] Until now, the beneficial effect of VEGF on podocytes was thought to be mediated indirectly by improvement of glomerular endothelial cell survival, because podocytes were not known to express VEGF receptors. However, in addition to this paracrine role of VEGF in the glomerulus, it is possible that VEGF has an autocrine function through its tyrosine-kinase receptor that is required for podocyte survival *in vitro*.[15,17] The data suggest that VEGF maintains podocyte function and survival by regulation of nephrin, possibly acting in an autocrine and/or paracrine manner in the progressive disease.

VEGF expression was downregulated via sFlt-1 in this particular model. A recent report demonstrated that signaling through extracellular matrix proteins, in particular, laminin and its receptor $\alpha 3\beta 1$ integrin, which is highly

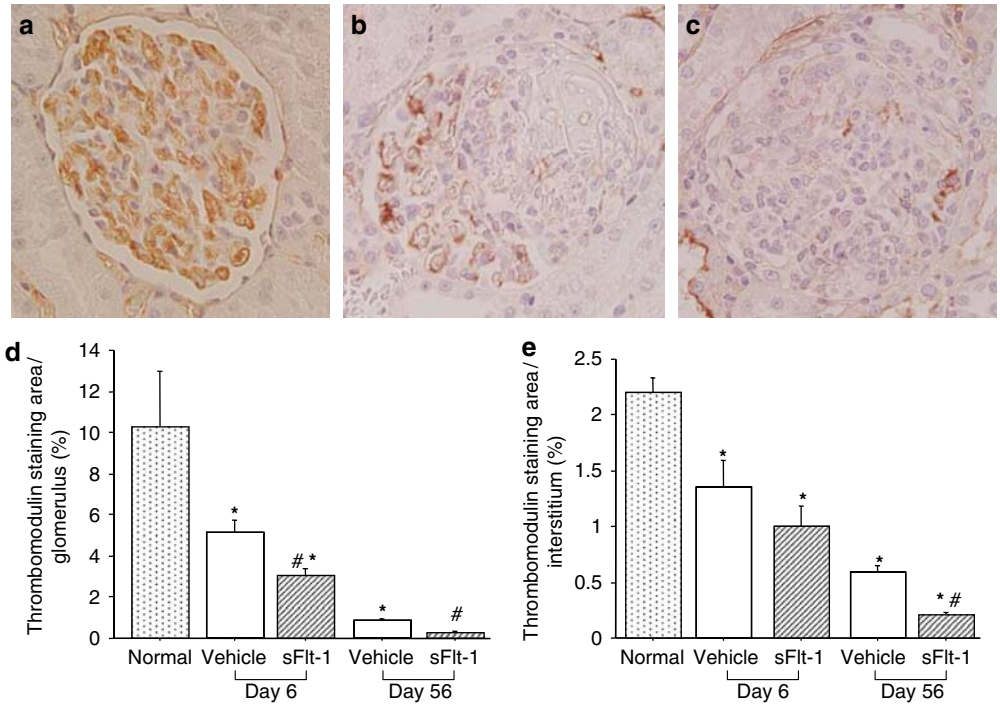

Figure 7 | VEGF inhibition accelerated glomerular and peritubular endothelial loss. (**a**) TM-positive endothelial cells were detected ubiquitously along with glomerular and peritubular capillaries in normal rats. (**b**) TM-positive endothelial cells were reduced in rats transfected with empty plasmid at day 6. (**c**) sFlt-1 treatment accelerated reduction of TM-positive endothelial cells at day 6. Original magnification, $\times$ 400. (**d**, **e**) Loss of TM-positive glomerular and peritubular endothelial cells was prominent by sFlt-1 treatment. $^{*}P<0.05$ compared with normal rats. $^{\#}P<0.05$ compared with vehicle-treated rats.

expressed in podocytes regulates VEGF production in cultured podocytes.[18] Hence, downregulated VEGF in this model, at least in part, is likely owing to the disturbance of the GBM matrix–podocyte interaction by anti-GBM antibodies. Further decrease in VEGF expression was observed by VEGF blockade via sFlt-1 during the course of disease. This might be explained by podocyte damage being augmented by inhibition of VEGF activity.[5,8,15,17] Therefore, downregulated VEGF in this model might perpetuate further podocyte loss and endothelial injury, thereby leading to glomerulosclerosis.

VEGF binds two related receptors, Flt-1 and KDR/Flk-1.[1,3] In rat kidney, Flk-1 has been shown to be expressed in glomerular and peritubular endothelial cells.[3] However, Flt-1 expression is controversial. In the present study, Flk-1 was locally expressed in glomerular and peritubular endothelial cells and was diminished during the disease course, concomitantly with progressive glomerular and PTC loss. Flt-1 was detected on podocytes in normal glomeruli, but was barely detected in diseased kidneys in this model. Therefore, Flk-1 appears to be the major mediator of endothelial cell mitogenesis and survival as well as angiogenesis in a crescentic GN, as reported previously.[19,20] PTC loss contributes to the etiology of the interstitial fibrosis by playing an essential role in impaired blood flow in tubular cells and interstitial cells. PTC loss may result from downregulated expression of VEGF in progressive renal diseases directly affecting PTC loss via its prosurvival effect on endothelial cells.[19,21] PTC loss may also be caused by filtered urinary proteins that lead to parenchymal damage and, eventually, renal fibrosis and dysfunction.[22] Therefore, PTC loss observed in sFlt-1-treated rats may play a crucial role in the deterioration of renal function. Recently, Flt-1 has been shown to be expressed on mesangial cells[23] and conditionally immortalized human podocyte cell line.[17] In addition, it is upregulated in certain disease models such as diabetic, passive Heymann nephritis, and puromycin aminonucleoside nephrosis.[3,24] Therefore, VEGF blockade via sFlt-1 accelerated the injury of endothelial cells as well as podocytes, mainly through the inhibition of Flk-1.

Monocytes/macrophages participate in inflammatory processes in crescentic GN. VEGF promotes monocyte chemotaxis via a primary effect on the receptor Flt-1.[25] In the present study, however, no significant effect of VEGF inhibition on crescentic formation, ED-1-positive macrophages, CD8-positive T-lymphocytes, or proliferating cell nuclear antigen-positive cells in the glomeruli was seen at day 6. These findings were consistent with the previous report that VEGF had no effect on infiltration of neutrophils, CD3-T lymphocytes, and ED-1-positive macrophages in the Thy-1/habu-snake venom GN model.[26] One plausible reason is that downregulated VEGF could not induce migration and activation of monocytes/macrophages, because VEGF-mediated chemotaxis for monocytes/macrophages depends on the dose and gradient of VEGF.[25] Therefore, although VEGF affects migration and activation of monocytes/macrophages when upregulated, such as in aortic and coronary vascular inflammation,[7,13,25,27] the residual VEGF

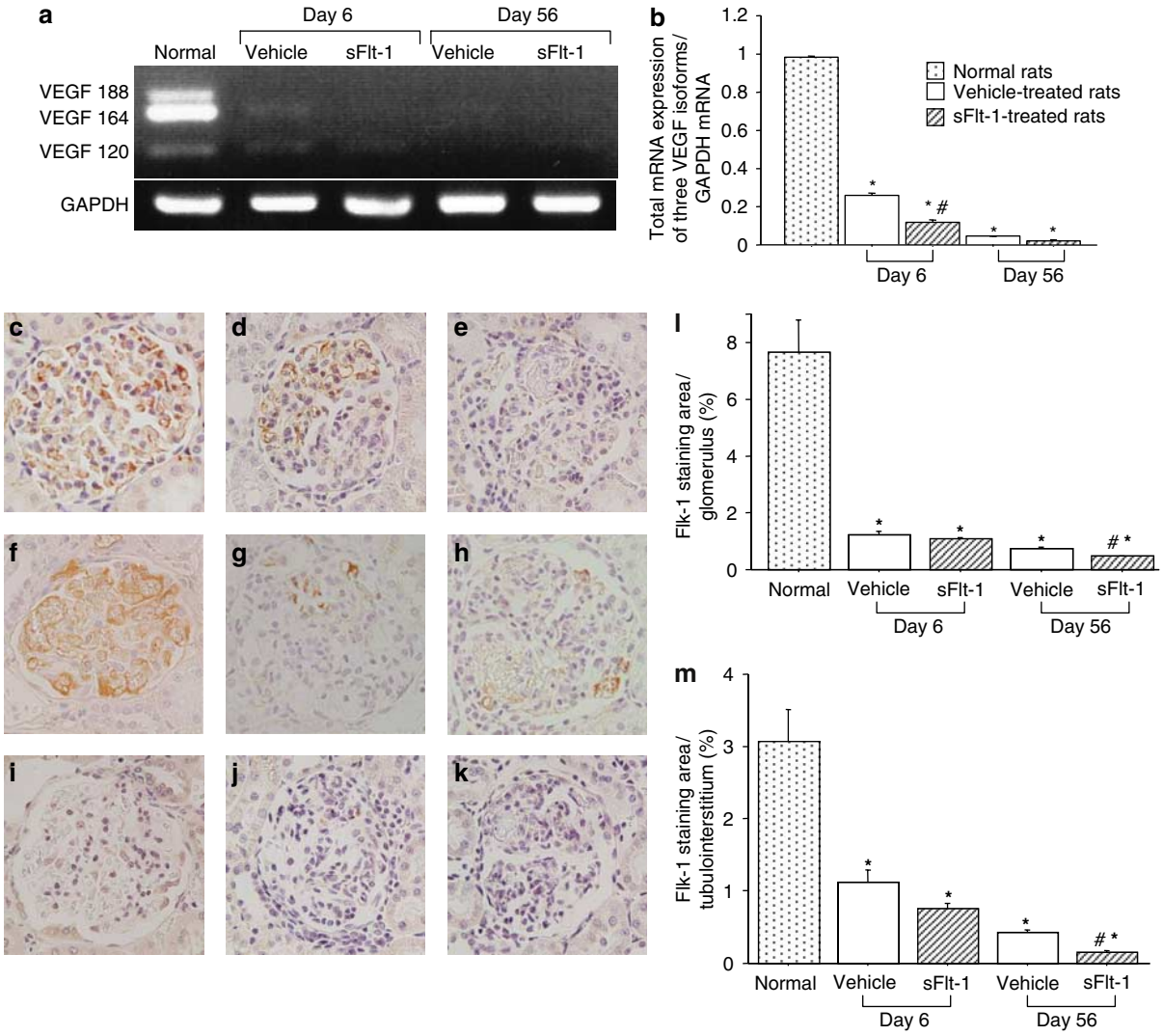

Figure 8 | Effects of anti-GBM GN and sFlt-1 treatment on expression of VEGF and VEGF receptors, Flt-1, and Flk-1. mRNA expression of VEGF in normal and diseased kidneys. (**a**) Expression of VEGF mRNA was decreased in anti-GBM GN, and further downregulated by sFlt-1 treatment. Densitometric analysis of data in (**a** and **b**). Total expression of three isoforms of VEGF mRNA in each sample is presented as a ratio to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA expression in that sample. $^{*}P<0.001$ compared with normal rats. $^{\#}P<0.001$ compared with vehicle-treated rats. (**c**) VEGF was detected in podocytes in normal kidneys. (**d**) In nephritic rats, VEGF expression was reduced, and (**e**) was further decreased by sFlt-1 treatment at day 6. (**f**) Flk-1 was expressed in glomerular and peritubular endothelial cells. (**g**, **l**, and **m**) Flk-1 expression was reduced in diseased kidneys, especially in glomeruli involved with crescentic lesions at day 6. (**h**, **l**, and **m**) Flk-1 expression was more severely decreased in sFlt-1-treated rats. Flt-1 was detected on podocytes in (**i**) normal glomeruli and was hardly expressed in diseased kidneys of both (**j**) vehicle-treated and (**k**) sFlt-1-treated rats. Original magnification, $\times 400$. $^{*}P<0.05$ compared with normal rats. $^{\#}P<0.05$ compared with vehicle-treated rats.

in this model might have no significant impact on monocytes/macrophages compared with its effects on endothelial and glomerular epithelial cells.

In conclusion, our study shows that VEGF plays a role in maintaining podocyte function as well as renal vasculature, thereby protecting glomeruli and interstitium from progressive renal insults.

MATERIALS AND METHODS

Animals

Inbred male Wistar–Kyoto rats, purchased from Charles River Japan Inc. (Atsugi, Kanagawa, Japan), aged 12 weeks were fed standard rat chow and given free access to water under 24 h light control. All the procedures used in the animal experiments complied with the standards set out in the Guideline for the Care and Use of Laboratory Animals in Takara-machi Campus of Kanazawa University.

Expression vector

The 3.3 kb mouse soluble Flt-1 gene, originally obtained from the mouse lung library,[28] was cloned into the *Bam*H1 (5′) and *Not*1 (3′) sites of the eukaryotic expression vector plasmid cDNA3.[7]

Preparation of anti-rat GBM antibodies

Rat GBM was prepared using the method of Krakower and Greenspon.[29] The preparation of anti-rat GBM antibodies was

described previously.[30] Specificity was confirmed by *in vitro* indirect immunofluorescence assays, using fluorescein isothiocyanate-conjugated anti-rabbit IgG, on frozen sections of normal Wistar rat kidneys. Sharp linear immunofluorescence was observed only along the GBM.

Experimental design

Twenty male Wistar–Kyoto rats were injected intravenously with 0.1 ml of nephrotoxic serum at day 0. Nine of 20 rats received an intramuscular injection of sFlt-1 plasmid (500 μg/150 μl phosphate-buffered saline) into the femoral muscles with a 26-G needle 3 days before injection of nephrotoxic serum, and five of nine rats received another injection of the plasmid 2 weeks thereafter. To enhance transgene expression, electroporation was performed at the injected site immediately after injection as described previously.[31,32] sFlt-1-treated rats were killed at days 6 (four rats) or 56 (five rats), and blood samples were collected. Muscle samples were obtained by partial excision of the sFlt-1-injected sites at days 6 and 56. Urine was collected 0, 6, 14, and 56 days after the nephrotoxic serum injection. The remaining 11 nephritic rats injected with empty plasmid with electroporation were also killed at days 6 (five rats) or 56 (six rats). As a control, urinary excretion of protein was measured at days 6, 14, 21, 28, and 56 in six normal rats transfected with sFlt-1 plasmid with electroporation. Levels of urinary protein excretion were determined at days 6, 14, and 56 in 10 normal untreated rats to establish normal values.

Expression of sFlt-1

The expression of sFlt-1 in skeletal muscle, which was fixed in 10% formalin followed by embedding in paraffin, was detected by immunohistochemistry using peroxidase-labeled polymer conjugated to goat anti-rabbit IgG (Envision System; DAKO Co., Carpinteria, CA, USA) and anti-Flt-1 antibodies (C-17; Santa Cruz Biotechnology, Santa Cruz, CA, USA) 6 days after disease induction.[31] Moreover, Flt-1 mRNA in femoral muscle was detected using RT-PCR. In brief, the cDNA was reverse-transcribed from 1 μg total RNA using a RT-PCR kit (Takara Shuzo, Tokyo, Japan). The cDNA product was amplified by PCR. Primers for mouse sFlt-1 (5′-GGTGCCCGCTCTTTG-3′ (sense); 5′-TGTCTCAGTGGGGATT GC-3′ (antisense)) were used to detect sFlt-1.[33] The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase was used for PCR controls.

Urinary sFlt-1 measurements

The commercially available enzyme-linked immunosorbent assay kits (R&D Systems, Minneapolis, MN, USA) were used to measure mouse urinary sFlt-1 according to the manufacturer's instructions.

Histopathological studies

A portion of renal tissue was prepared as reported previously.[30] The total number of cells and number of glomeruli with crescentic formation were measured and expressed as described previously.[30] The extent of glomerular sclerosis was expressed as a percentage of the periodic acid-methanamine silver-positive area/whole glomerular area as described previously.[34] Each area was measured by a computer-aided manipulation using Mac Scope version 6.02 (Mitani Shoji Co., Ltd, Fukui, Japan). The extent of interstitial fibrosis, expressed as blue in Mallory–azan staining, was evaluated as reported previously.[32,35]

In an indirect immunoperoxidase staining, endothelial cells were detected with mouse anti-TM monoclonal antibody (141C01; NeoMarkers, Fremont, CA, USA), injured podocytes with mouse anti-desmin monoclonal antibody (D33; DAKO Co.), VEGFR-1 with goat anti-Flt-1 polyclonal antibodies (C-17; Santa Cruz Biotechnology), and VEGFR-2 with mouse anti-Flk-1 monoclonal antibody (A-3; Santa Cruz Biotechnology). In each biopsy, TM- and Flk-1-positive areas in glomeruli and tubulointerstitium were identified and expressed in a similar manner as described above, respectively.[34,35]

Another portion of renal tissue was frozen rapidly and immunostained directly with fluorescein isothiocyanate-conjugated, antiproliferating cell nuclear antigen (No. 033L245; Leinco Technologies Inc., St Louis, MO, USA) or indirectly with a mouse monoclonal antibody against rat tissue monocytes and macrophages, ED1 (IgG1; BMA Biomedicals Ltd, Augst, Switzerland) or a mouse monoclonal antibody against rat CD8 (IgG1; No. 0412; Cedarlane, Hornby, Ontario, Canada). Positive cells were counted on at least 50 randomly chosen glomeruli. Renal tissues obtained from four normal Wistar–Kyoto rats were used as negative controls. To evaluate the induction of GN, fluorescein isothiocyanate-conjugated anti-rabbit IgG (No. 38236; Organon Teknika Corporation, Durham, NC, USA), fluorescein isothiocyanate-conjugated anti-rat C3 (No. 38810; Organon Teknika Corporation), and fluorescein isothiocyanate-conjugated anti-rat IgG (No. 38731; Organon Teknika Corporation) were used.[30]

Detection of VEGF protein and its mRNA

To examine the production of VEGF in kidney before and after disease induction, immunohistochemical analysis was performed on paraffin-embedded tissue specimens with mouse anti-VEGF monoclonal antibody (C-1; Santa Cruz Biotechnology). In addition, to determine transcripts of VEGF, RT-PCR was performed using primers for VEGF (5′-GACCCTGGTGGACATCTTCCAGGA-3′ (sense); 5′-GGTGAGAGGTCTAGTTCCCGA-3′ (antisense)) with expected sizes of 514, 462, and 330 bp for amplification of VEGF188, VEGF 164, and VEGF 120, respectively.[36]

Detection of the glomerular epithelial slit diaphragm-associated molecules and extent of foot process effacement

Morphological changes of podocytes were examined by electron microscopy as described previously (Hitachi H-600, Tokyo, Japan).[30] Next, the presence of nephrin was demonstrated immunohistochemically in frozen tissue specimens with mouse anti-rat nephrin monoclonal antibody 5-1-6, and that of podocalyxin with mouse anti-podocalyxin monoclonal antibody (4D5; gift from Dr Masanori Hara).

mRNA expression of nephrin, podocin, and podocalyxin was analyzed by real-time RT-PCR on isolated cortices of kidneys from each group of rats. The cDNA was reverse-transcribed from 1 μg total RNA using SuperScript II RNase H^- reverse transcriptase (Invitrogen, Carlsbad, CA, USA). Reverse transcription was performed using the following parameters: 10 min at 25°C, 30 min at 48°C, and 5 min at 95°C. In real-time PCR experiments, the Sequence Detection System (7900HT; Applied Biosystems, Foster City, CA, USA) was used. Primers for nephrin (Rn00575235, Nphs1; Applied Biosystems), podocin (Rn00709834, Nphs2; Applied Biosystems), and podocalyxin (Rn00593804, Podxl; Applied Biosystems) were used. The housekeeping gene, β-actin (Rn00667869, Actb; Applied Biosystems) was used for PCR controls. The reactions were incubated at 95°C for 10 min, followed by 40 cycles of 15 s at 95°C, 10 s at 55°C, and 20 s at 72°C. mRNA expression of nephrin,

podocin, and podocalyxin in each sample was finally described after correction with β-actin expression. In addition, expression of podoplanin mRNA was analyzed by RT-PCR. cDNA was reverse-transcribed from 1 μg total RNA using a reverse transcription-PCR kit (Takara Shuzo). Primers for podoplanin (forward: 5′-GAGCG TTTGGTTTCTGGGACTCA-3′; reverse: 5′-GGTGAGAGGTCTAGT TCCCGA-3′)[37] were used. The housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase was used for PCR control. The reactions were incubated at 94°C for 3 min, followed by 35 cycles of 30 s at 94°C, 30 s at 58°C, and 60 s at 72°C.[37] Scanner analysis was examined as described previously.[32,35]

Western blot analysis

Isolated cortical portions of kidneys from each group were prepared as described elsewhere. The protein concentration of each sample was measured by Bradford Protein Assay kit (Bio-Rad Laboratories Inc., Hercules, CA, USA). The homogenate was diluted with Laemmli sample buffer (Bio-Rad Laboratories Inc.) and was boiled for 5 min and cooled on ice. Ten micrograms of protein were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electroblotted onto a polyvinylidene difluoride membrane (Bio-Rad Laboratories Inc.). The membrane was blocked with bovine serum albumin-containing TBST buffer (20 mM Tris, pH 7.6, 140 mM NaCl, and 0.1% Tween 20), and then incubated with goat anti-nephrin polyclonal antibodies (N-20; Santa Cruz Biotechnology)[38] or rabbit anti-podocalyxin polyclonal antibodies (KR064; Transgenic Co., Hyogo, Japan). To visualize the signals, the membrane was incubated with biotinylated rabbit anti-goat immunoglobulins (DAKO Co.) followed by streptavidin–horseradish peroxidase complex (DAKO Co.) for nephrin or with peroxidase-labeled polymer conjugated to goat anti-rabbit IgG (Envision System; DAKO Co.) for podocalyxin.

Determination of urinary protein, blood urea nitrogen, serum creatinine, and serum total protein concentrations

Urinary protein concentrations were determined as reported previously.[39] Urinary protein excretion was expressed as the ratio of urinary protein to urinary creatinine. Blood urea nitrogen, serum and urinary creatinine, and serum total protein levels were measured using an automated analyzer (Hitachi, Tokyo, Japan) according to the manufacturer's instructions.

Statistical analyses

The mean and standard error were calculated on all parameters determined in this study. Statistical analyses were performed using the unpaired Student's *t*-test and Kruskal–Wallis test. Values of $P<0.05$ were considered statistically significant.

ACKNOWLEDGMENTS

We gratefully thank Drs. Raghu Kalluri, Hikaru Sugimoto (Harvard Medical School), and Dr Joan Sechler (National Institute of Health) for their critical review of this paper. TW is a recipient of a Grant-in-Aid from the Ministry of Education, Science, Sports, and Culture in Japan. This work is supported in part by a Grant-in-Aid from the Ministry of Health, Labor, and Welfare of Japan.

REFERENCES

1. Ferrara N. Role of vascular endothelial growth factor in the regulation of angiogenesis. *Kidney Int* 1999; **56**: 794–814.
2. Simon M, Rockl W, Hornig C *et al.* Receptors of vascular endothelial growth factor/vascular permeability factor (VEGF/VPF) in fetal and adult human kidney: localization and [^{125}I] VEGF binding sites. *J Am Soc Nephrol* 1998; **9**: 1032–1044.
3. Schrijvers BF, Flyvbjerg A, DeVriese AS. The role of vascular endothelial growth factor (VEGF) in renal pathophysiology. *Kidney Int* 2004; **65**: 2003–2017.
4. Kitamoto Y, Tokunaga H, Tomita K. Vascular endothelial growth factor is an essential molecule for mouse kidney development: glomerulogenesis and nephrogenesis. *J Clin Invest* 1997; **99**: 2351–2357.
5. Eremina V, Sood M, Haigh J *et al.* Glomerular-specific alterations of VEGF-A expression lead to distinct congenital and acquired renal diseases. *J Clin Invest* 2003; **111**: 707–716.
6. Maynard SE, Min JY, Merchan J *et al.* Excess placental soluble fms-like tyrosine kinase 1 (sFlt-1) may contribute to endothelial dysfunction, hypertension, and proteinuria in preeclampsia. *J Clin Invest* 2003; **111**: 649–658.
7. Zhao Q, Egashira K, Inoue S *et al.* Vascular endothelial growth factor is necessary in the development of arteriosclerosis by recruiting/activating monocytes in a rat model of long-term inhibition of nitric oxide synthesis. *Circulation* 2002; **105**: 1110–1115.
8. Sugimoto H, Hamano Y, Charytan D *et al.* Neutralization of circulating Vascular endothelial growth factor (VEGF) by anti-VEGF antibodies and soluble VEGF receptor 1 (sFlt-1) induces proteinuria. *J Biol Chem* 2003; **278**: 12605–12608.
9. Kabbinavar F, Hurwits HI, Fehrenbacher L *et al.* Phase II, randomized trial comparing bevacizumab plus fluorouracil (FU)/leucovorin (LV) with FU/LV alone in patients with metastatic colorectal cancer. *J Clin Oncol* 2003; **21**: 60–65.
10. Yang JC, Haworth L, Sherry RM *et al.* A randomized trial of bevacizumab, an anti-vascular endothelial growth factor antibody, for metastatic renal cancer. *N Engl J Med* 2003; **349**: 427–434.
11. Hurwitz H, Fehrenbacher L, Novotny W *et al.* Bevacizumab plus irinotecan, fluorouracil, and leucovorin for metastatic colorectal cancer. *N Engl J Med* 2004; **350**: 2335–2342.
12. Pavenstadt H, Kriz W, Kretzler M. Cell biology of the glomerular podocyte. *Physiol Rev* 2003; **83**: 253–307.
13. Zhao Q, Ishibashi M, Hiasa K *et al.* Essential role of vascular endothelial growth factor in angiotensin II-induced vascular inflammation and remodeling. *Hypertension* 2004; **44**: 1–7.
14. Kestila M, Lenkkeri U, Mannikko M *et al.* Positionally cloned gene for a novel glomerular protein-nephrin is mutated in congenital nephrotic syndrome. *Mol Cell* 1998; **1**: 575–582.
15. Foster RR, Saleem MA, Mathieson PW *et al.* Vascular endothelial growth factor and nephrin interact and reduce apoptosis in human podocytes. *Am J Physiol Renal Physiol* 2005; **288**: F48–F57.
16. Shimizu A, Masuda Y, Mori T *et al.* Vascular endothelial growth factor165 resolves glomerular inflammation and accelerates glomerular capillary repair in rat anti-glomerular basement membrane glomerulonephritis. *J Am Soc Nephrol* 2004; **15**: 2655–2665.
17. Rebecca RF, Rachel H, Karen A *et al.* Functional evidence that vascular endothelial growth factor may act as an autocrine factor on human podocytes. *Am J Physiol Renal Physiol* 2003; **284**: F1263–F1273.
18. Datta K, Li J, Karumanchi SA *et al.* Regulation of vascular permeability factor/vascular endothelial growth factor (VPF/VEGF-A) expression in podocytes. *Kidney Int* 2004; **66**: 1471–1478.
19. Ohashi R, Shimizu A, Masuda Y *et al.* Peritubular capillary regression during the progression of experimental obstructive nephropathy. *J Am Soc Nephrol* 2002; **13**: 1795–1805.
20. Yuan HT, Tipping PG, Li XZ *et al.* Angiopoeitin correlates with glomerular capillary loss in anti-glomerular basement membrane glomerulonephritis. *Kidney Int* 2002; **61**: 2078–2089.
21. Kang DH, Joly AH, Oh SW *et al.* Impaired angiogenesis in the remnant kidney model: I. Potential role of vascular endothelial growth factor and thrombospondin-1. *J Am Soc Nephrol* 2001; **12**: 1434–1447.
22. Remuzzi G, Bertani T. Pathophysiology of progressive nephropathies. *N Engl J Med* 1998; **339**: 1448–1456.
23. Thomas S, Vanuystel J, Gruden G *et al.* Vascular endothelial growth factor receptors in human mesangium *in vitro* and in glomerular disease. *J Am Soc Nephrol* 2000; **11**: 1236–1243.
24. Kanellis J, Levidiotis V, Khong T *et al.* A study of VEGF and its receptors in two rat models of proteinuria. *Nephron Physiol* 2004; **96**: 26–36.
25. Barleon B, Sozzani S, Zhou D *et al.* Migration of human monocytes in response to vascular endothelial growth factor (VEGF) is mediated via the VEGF receptor flt-1. *Blood* 1996; **87**: 3336–3343.
26. Masuda Y, Shimizu A, Mori T *et al.* Vascular endothelial growth factor enhances glomerular capillary repair and accelerates resolution of experimentally induced glomerulonephritis. *Am J Pathol* 2001; **159**: 599–608.

27. Celletti FL, Waugh JM, Amabile PG *et al.* Vascular endothelial growth factor enhances atherosclerotic plaque progression. *Nat Med* 2001; **7**: 425–429.
28. Kondo K, Hiratsuka S, Subbalakshmi E *et al.* Genomic organization of the flt-1 gene encoding for vascular endothelial growth factor (VEGF) receptor-1 suggests an intimate evolutionary relationship between the 7-Ig and the 5-Ig tyrosine kinase receptors. *Gene* 1998; **208**: 297–305.
29. Krakower C, Greenspon SA. Localization of nephrotoxic antigen within the isolated renal glomerulus. *Arch Pathol* 1951; **51**: 629–639.
30. Wada T, Yokoyama H, Furuichi K *et al.* Intervention of crescentic glomerulonephritis by antibodies to monocyte chemotactic and activating factor (MCAF/MCP-1). *FASEB J* 1996; **10**: 1418–1425.
31. Furuichi K, Wada T, Iwata Y *et al.* Gene therapy expressing amino-terminal truncated monocyte chemoattractant protein-1 prevents renal ischemia-reperfusion injury. *J Am Soc Nephrol* 2003; **14**: 1066–1071.
32. Wada T, Furuichi K, Sakai N *et al.* Gene therapy via blockade of monocyte chemoattractant protein-1 for renal fibrosis. *J Am Soc Nephrol* 2004; **15**: 940–948.
33. Nalbandian A, Dettin L, Dym M, Ravindranath N. Expression of vascular endothelial growth factor receptors during male germ cell differentiation in the mouse. *Biol Reprod* 2003; **69**: 985–994.
34. Ando Y, Moriyama T, Miyazaki M *et al.* Enhanced glomerular expression of caldesmon in IgA nephropathy and its suppression by glucocorticoid-heparin therapy. *Nephrol Dial Transplant* 1998; **13**: 1168–1175.
35. Kitagawa K, Wada T, Furuichi K *et al.* Blockade of CCR2 ameliorates progressive fibrosis in kidney. *Am J Pathol* 2004; **165**: 237–246.
36. Kim NH, Jung HH, Cha DR, Choi DS. Expression of vascular endothelial growth factor in response to high glucose in rat mesangial cells. *J Endocriol* 2000; **165**: 617–624.
37. Han GD, Koike H, Nakatsue T *et al.* IFN-inducible protein-10 has a differential role in podocyte during Thy1.1 glomerulonephritis. *J Am Soc Nephrol* 2003; **14**: 3111–3126.
38. Hou YX, Cui L, Riordan JR, Chang XB. ATP binding to the first nucleotide-binding domain of multidrug resistance protein MRP1 increases binding and hydrolysis of ATP and trapping of ADP at the second domain. *J Biol Chem* 2002; **277**: 5110–5119.
39. Wada T, Tomosugi N, Naito T *et al.* Prevention of proteinuria by the administration of anti-interleukin 8 antibody in experimental acute immune complex-induced glomerulonephritis. *J Exp Med* 1994; **180**: 1135–1140.